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Introduction

The long-term goal of this research project is to develop CCR5 antagonists with the structure feature of anibamine as novel anti prostate cancer agents. The tasks of this project include: 1. Chemical synthesis of the ligand designed as anibamine derivatives; 2. Radio-ligand competition binding assay to evaluate the binding affinity of the ligands synthesized; 3. Characterization of CCL5 and CCR5 expression in SV40T-immortalized human prostate epithelial cell lines of the same cellular lineage with varying tumorigenicity and metastatic capacity in vivo (P69, M2182, M2205 and M12); 4. Investigation of the impact of chemokine receptor CCR5 antagonist on prostate cancer cell growth and progression using M12, PC-3, and DU-145 cell lines; 5. Molecular modeling study. In order to achieve these aims, we have first designed and synthesized a series of anibamine analogs based on "Deconstruction-Reconstruction-Elaboration" method. In the second year of this project, additional twenty novel ligands have been prepared as derivatives of anibamine for biological screening assays. Second, several progressive biological assays to evaluate the anti prostate cancer activity of the ligands have been set up and pursued. Primarily, a Ca^{2+} mobilization assay has been set up and the protocol has been tested successfully. Some of known ligands and new ligands in our library have been tested in the assay. Second, a RANTES-binding inhibition assay protocol has been set up and some new ligands have been tested in this assay. Additionally, biological screening includes the test of capacity of these novel compounds to inhibit proliferation and/or apoptosis by the human prostate cancer cell lines M12, PC-3, and DU-145 has been conducted continuously to evaluate the efficacy of more ligands. A molecular modeling study (3D QSAR) protocol has been set up and tested with some representative ligands in our library. Combined the result of the expression of CCL5 and CCR5 in human prostate epithelial cell lines of the same cellular lineage but with differing in vivo phenotypes (P69SV40TAg, M2182, M2205, and M12), one manuscript focusing on the comprehensive characterization of anibamine as anti prostate cancer agent has been accepted for publication in *Bioorganic and Medicinal Chemistry Letters*.

Annual Progress Report

The long-term goal of this research project is to develop CCR5 antagonist with the structure feature of anibamine as novel anti prostate cancer agents. Based on the approved Statement of Work (SOW) of this award, the following tasks were planned originally to be accomplished within two-year period of time:

Task 1. Chemical synthesis of the ligand designed as anibamine derivatives

Task 2. Radio-ligand competition binding assay to evaluate the binding affinity of the ligands synthesized

Task 3. Characterization of CCL5 and CCR5 expression in SV40T-immortalized human prostate epithelial cell lines of the same cellular lineage with varying tumorigenicity and metastatic capacity in vivo

Task 4. Investigation of the impact of chemokine receptor CCR5 antagonist on prostate cancer cell growth and progression using M12, PC-3, DU-145 and LNCaP cell lines

Task 5. Molecular modeling study

In the first year all five tasks had been pursued accordingly and the results were summarized, submitted and reviewed. In the second year we again kept conducting our research based on the approved SOW and made significant progress. Right now the award is under No-Cost Extension period (06/01/2010 to 06/30/2011). The research outcome in the second year of performance is summarized as below:

Task 1. Chemical synthesis of the ligand designed as anibamine derivatives

Our laboratory has accomplished the total synthesis of anibamine and published the results in *Organic Letters*, 2007, 9(10), 2043-6. This biosynthetic approach is being applied directly to our current chemical synthesis of the newly designed derivatives of anibamine. The initial structural modification of anibamine is following the "Deconstruction-Reconstruction-Elaboration" method, as proposed in Figure 1. In this second year project period, we further explored the method more exhaustedly in order to define the structure-activity-relationship of anibamine comprehensively.

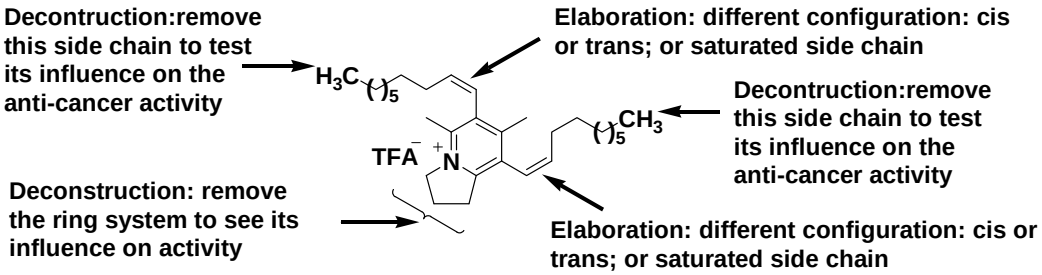
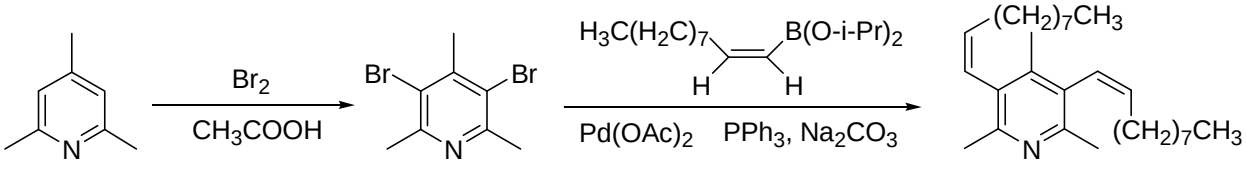


Figure 1. Initial structure modification of anibamine

1.1 Ring System Deconstruction

The central ring system of anibamine largely determines the molecular conformation of anibamine. Thus, by removing the fused ring, the conformation of the molecule will be changed. Shown in scheme 1, the synthesis of another non-ring derivative of anibamine was accomplished.



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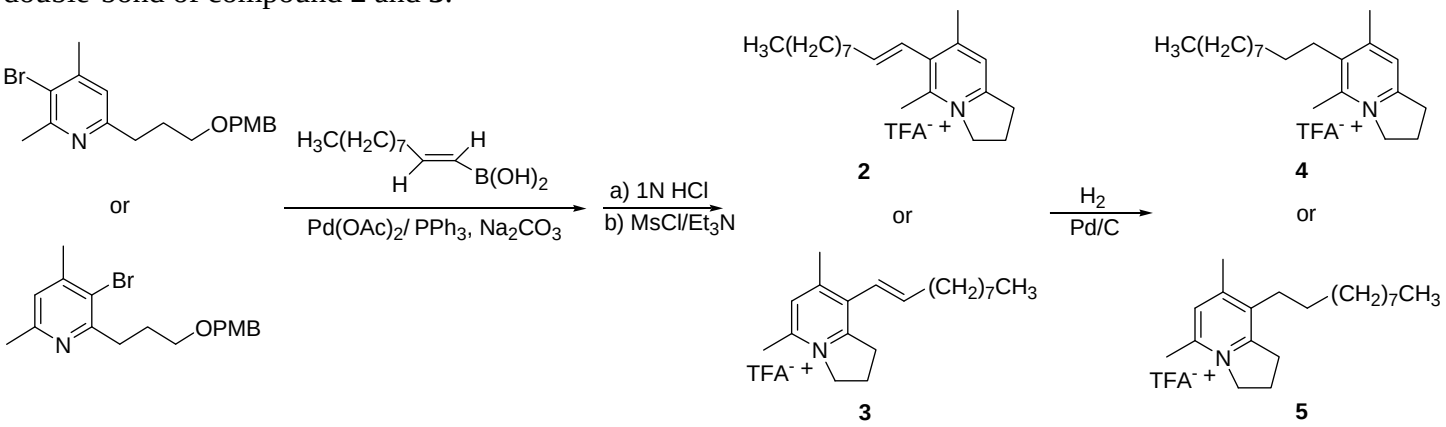
Scheme 1. The synthesis of non-ring derivative of anibamine

1.2 Elaboration procedure

Further deconstruction/elaboration products were designed based on anibamine main skeleton to exhaust the structural variants of anibamine. The purpose of this study includes: one, the chemical synthesis of these new

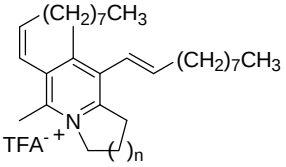
ligands provides synthetic methodologies to future preparation of more structure-diversified anibamine derivatives; two, the biological screening study on these ligands will help identify any possible missed structure features that are critical to anti cancer activity from our previous studies.

Compound **2** and **3** will test the combination of deconstruction and elaboration by removing one side chain in anibamine while changing the stereochemistry of the double bond on the other one. The chemical synthesis of compound **2** and **3** is shown in scheme **2** while compound **4** and **5** were prepared simply by reducing the double-bond of compound **2** and **3**.

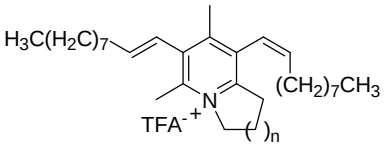


Scheme 2. The chemical synthesis of compound 2 to 5.

Compound **6** to **11** were designed to see if changing the configuration of double bond on only one side chain of anibamine will influence the anti cancer activity.

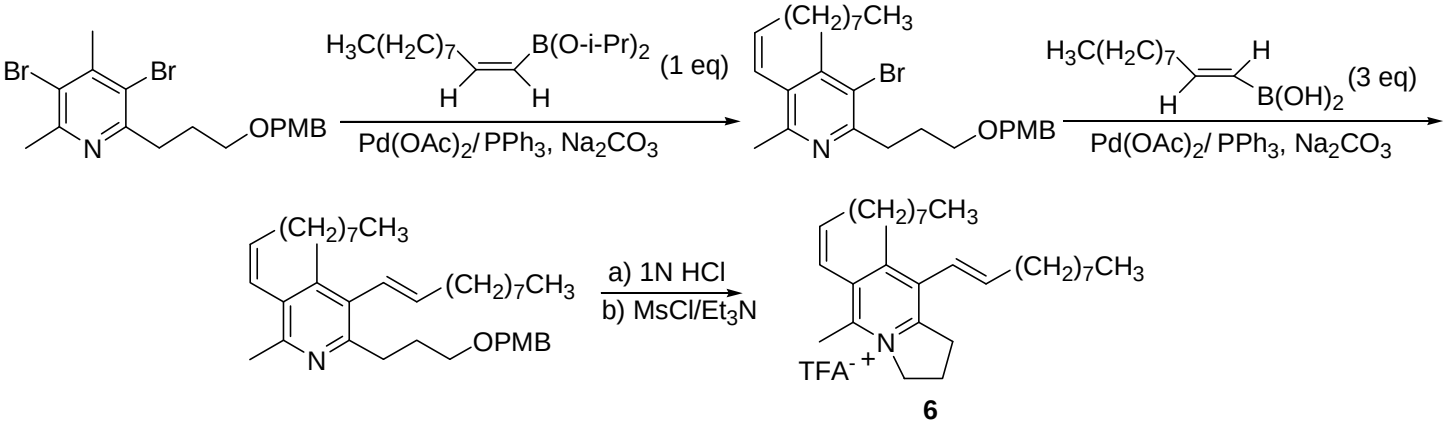


n = 1, compound **6**
n = 2, compound **7**
n = 3, compound **8**



n = 1, compound **9**
n = 2, compound **10**
n = 3, compound **11**

The chemical synthesis of compound **6** through **11** required regio- and stereo- selective introduction of trans- and cis- double bond moiety. The stereo-selectivity were achieved by applying Suzuki's Coupling Reaction, while the regio-selectivity were achieved by taking advantage of the steric hindrance of the 2-position side chain, as shown in scheme **3** using compound **6** as an example.

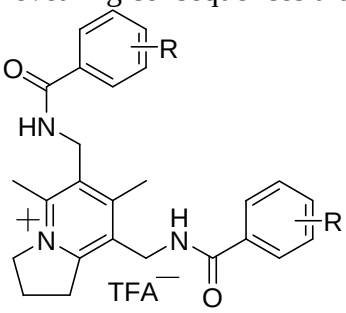


Scheme 3. The chemical synthesis of compound 6

1.3 Further side chain modification

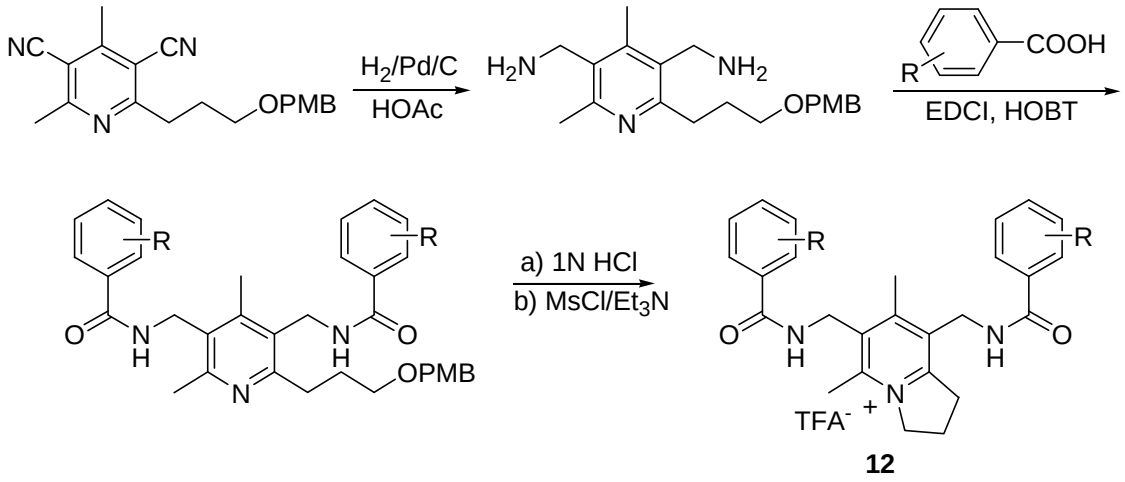
We decided to modify the side chains of anibamine more dramatically. The reasons are, at first, the calculated log K_{ow} for anibamine is 9.1, which indicates that its lipophilicity is significantly higher than the value set forth by "Lipinski's rule of 5" for drug-like compounds. Second, in comparing the chemical structure of anibamine with other known CCR5 antagonists, a major difference is that their two side chains are simple, undecorated,

aliphatic chains. Introduction of more chemical structure features on these side chains will likely have very revealing consequences that will effectively drive this structure-activity relationship study.



Therefore, an amide bond was first used as linkage for the side chain to introduce possible hydrogen bonding property and to utilize its partial double bond character. Then, an aromatic moiety (substituted phenyl ring system at this stage) were introduced to simulate the aliphatic chain of anibamine with similar number of carbon atoms. The substitutions on the phenyl ring were sequentially introduced by following Topliss Operation Scheme ("**Topliss Tree**"), as shown in sequence of H, 4-Cl, 4-OMe, 4-Me, 3,4-Cl_2, 3-Cl, 4-tBu, 4-CF_3, 3-CF_3. Total **9** new compounds have been synthesized.

The chemical synthetic routes to pursue these ligands have been formulated and shown in scheme 4.



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Scheme 4. The chemical synthetic routes to synthesize novel ligands.

In summary, total of **twenty** novel ligands as anibamine derivatives have designed and synthesized through multi-step chemical synthesis in the second year. They all have been fully characterized by NMR, IR, and MS. Elementary analysis was also conducted for each compound for final verification of their chemical composition.

Task 2. Radio-ligand competition binding assay to evaluate the binding affinity of the ligands synthesized

In the first year we setup a radio-ligand binding assay protocol to conduct the RANTES binding inhibition assays to test the CCR5 receptor affinity of all the compounds synthesized. The assay protocol has been validated. The shortcomings of this assay include that the radio-ligand [^{125}I]RANTES is a radio-safety harzard, this radio-ligand is costly and short-life, and it is difficult to realize high-throughput screening.

Therefore, in order to overcome these shortcomings, we also developed a Ca^{2+} mobilization assay as the pre-screening method in the second year. This Ca^{2+} mobilization assay has been applied in the binding affinity and function characterization of GPCRs ligands successfully in the literature. Specifically it has been adopted in the chemokine receptor CCR5 antagonists development. The advantages of Ca^{2+} mobilization assay over the traditional radio-ligand binding assay and GTPγS functional assay are economic, efficient, high-throughput, and environment-friendly.

2.1 Ca^{2+} mobilization assay

We developed the Ca^{2+} mobilization assay protocol in our lab and tested against some known CCR5 antagonists. As shown in Figure 2, CCR5 agonist CCL5/RANTES gave full stimulation of Ca^{2+} release while CCR5 antagonist Maraviroc showed dose-response inhibition of RANTES stimulation of Ca^{2+} mobilization. Similar results were observed with another CCR5 antagonist TAK-779. When anibamine was tested, it also showed potent antagonism with a much higher IC_{50} value. The results are summarized in Table 1. Interestingly, the IC_{50} values of these antagonists correlated nicely with their binding affinity reported in literature, which indicates that Ca^{2+} mobilization assay may be also applied to evaluate their binding affinity to CCR5 indirectly.

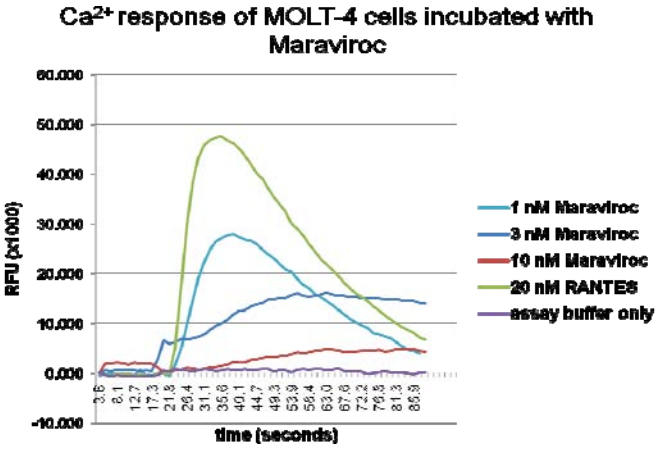


Table 1. Ca^{2+} mobilization assay results for known CCR5 antagonists

Compound	IC_{50} in Ca^{2+} mobilization	IC_{50} in affinity binding against RANTES
Maraviroc	1.57 nM	5.2 nM
TAK-779	1.48 nM	1.4 nM
Anibamine	1.20 uM	1.0 uM

Figure 2. Ca^{2+} mobilization assay results.

While we mainly look for CCR5 antagonists, the ligands were first tested with various doses for possible agonist activity. The protocol is the same for antagonism study, except no CCL5 (RANTES) will be added.

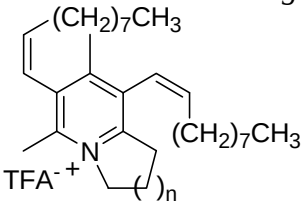
In the antagonism assay, CCL5/RANTES at 20 nM alone were used as agonist control, Maraviroc at 1 nM were used as antagonist positive control while DMSO were used as negative control.

Cell Culture Conditions: Cells are cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. The base medium for this cell line is ATCC-formulated RPMI-1640 Medium with 10% FBS, 1% L-Glu and 0.1% geneticin. Media needs to be added every 2-3 days and the cell concentration needs to be kept below 2,000,000 cells/ml.

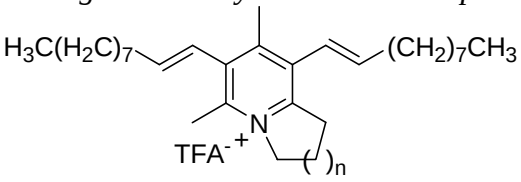
Experimental Protocol: MOLT-4 cells were plated in black 96-well plates with transparent bottom (Greinier Bio-one) at 100,000 cells per well in 50:1 HBSS:HEPES assay buffer. They were incubated for 1 hour at 37º and 5% CO_2 with control buffer or varying concentration of antagonist for a total volume of 130 µL per well. Cells were then incubated with 50 µL of Fluo-4-AM loading buffer (40 µL 2 µM Fluo-4 dye, 100 µL 2.5 mM probenacid, in 5 mL assay buffer) for an additional hour. 20 µL 200 nM RANTES solution in assay buffer or assay buffer alone were added to the wells and changes in Ca^{2+} concentration were monitored for 1 minute using a multichannel plate reader (FlexStation 3, Molecular Devices). Fifteen seconds before addition of chemokine or buffer were measured to detect background fluorescence. Normalization of data obtained from each fluorescence trace was performed by the following equation: (peak fluorescence - basal fluorescence)/basal fluorescence, where peak fluorescence is the highest peak in fluorescence after addition of chemokine and basal fluorescence is the average of the basal fluorescence in the first 15 seconds of the assay.

IC_{50} calculation: The half maximal inhibitory concentrations (IC_{50}) of compounds were determined with Prism 2 (GraphPad software) by constructing their dose-response curves to the logistic equation.

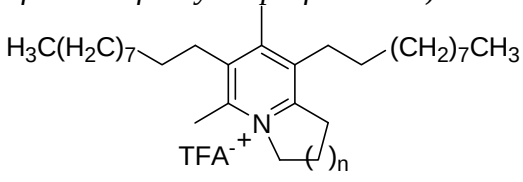
Screening results as follows (*For discussion convenience, the numbering system for Task 2 is different from Task 1 since the biological screening results may include the compounds from the first year performance*):



n = 1, anibamine **1**
n = 2, compound **2**
n = 3, compound **3**



n = 1, compound **4**
n = 2, compound **5**
n = 3, compound **6**



n = 1, compound **7**
n = 2, compound **8**
n = 3, compound **9**

Table 2. Ca^{2+} mobilization screening results.

Compounds	1	2	3	4	5	6	7	8	9
IC50 (Ca^{2+}) uM	0.65	0.84	0.77	2.76	2.15	1.56	2.7	>10	7.7

From Table 2 we can see that the ring size is critical for the binding affinity of these ligands to the receptor.

2.2 Radio-ligand binding assay

Once the ligands are characterized in Ca^{2+} mobilization assay, the ones that show IC50 value lower than 1 uM were further tested in the [^{125}I]RANTES radio-ligand binding assay to characterize their binding affinity to CCR5 receptor directly.

To test the CCR5 receptor affinity of the compounds synthesized, Chinese hamster ovary (CHO) cells expressing human CCR5 were used in the binding experiments. The experiment were conducted at room temperature in the presence of [^{125}I]RANTES. IC_{50} values (the concentration required to inhibit the binding of [^{125}I]RANTES by 50%) were used to evaluate the relative affinity of the compounds to CCR5. Normally, compounds with an IC_{50} concentration at 10μM or lower are considered active and will be further evaluated in additional in vitro assays. The binding assay will provide preliminary data for further molecular design, especially as guided by molecular modeling where downstream biological effects are not explicitly considered.

2.2.1 CCR5/CHO Cell Transfection CHO Cells are transfected with CCR5 plasmid DNA (UMR cDNA Resource Center) using LipofectAMINE Reagent (GibcoBRL) according to the manufacturer's recommendation. Briefly, a lipid/DNA mixture is prepared in 200 µL of serum-free media (3 µL lipid and 1 µg DNA) and incubated for 45 min at 22°C. Serum-free media is then added to bring the final volume to 1 mL, which is then added to each well of cells. After 3 hr, the lipid/DNA mixture is removed and replaced with 1 mL of FBS-containing medium. For selection of stable cell lines, the media is replaced with selection media (containing 4 to 6 mg/mL of G418) after 36 hr. These cells are then split into 96 well plates at a concentration of 1 cell per three wells. The entire time the cell are being grown in the presence of 1 mg/mL of G418 to continue to maintain the selection process. Once the cells get confluent, Western blots will be conducted for each clone utilizing a human CCR5 polyclonal antibody. The cloned cells with CCR5 expression will be adopted to further cell culture.

2.2.2 Cell Culture and Drug Treatments Cells are cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. The medium is a 1:1 mixture of DMEM and Nutrient Mixture F12 containing 5% fetal bovine serum, 100 units/mL each of penicillin and streptomycin. Transfected cell medium also contains 0.4 mg/mL G-418.

2.2.3 Membrane Homogenate Preparation Cells are harvested by replacing the media with PBS + 0.4% EDTA and collected by centrifugation at 1000 x g. Samples are homogenized in 20 volumes ice-cold membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) with a Polytron. The homogenate is centrifuged at 48,000 x g for 10 min, resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, pH 7.4), centrifuged again as above, and resuspended in assay buffer. Protein values are determined by the Bradford method.

2.2.4 CCR5 Receptor Binding Saturation binding is performed by incubating membranes for 90 minutes at 30°C with 0.5-15 nM [^{125}I]RANTES in assay buffer in a 0.5 mL volume. Non-specific binding is determined with 200 nM RANTES. For competition assays, membranes are incubated as above with 2 nM [^{125}I]RANTES and various concentrations of unlabeled ligand, to determine competitor affinity for CCR5. The reaction is terminated by rapid filtration (GF-C filter paper is presoaked for at least half hour in 0.3% PEI solution). Bound radioactivity is determined by gamma counter for [^{125}I] immediately [54].

2.2.5 Data analysis For competition binding assay, linear regression analysis of Hill plots (plot of log[B / (B_0 - B)] as a function of log[X]; where B is the pmol/mg of radioligand bound and X is the concentration of unlabeled competitor) will be conducted to determine the IC_{50} value for each ligand. "B" will be calculated as % of radioligand bound at each concentration of competitor and B_0 will be normalized to be 100%. The IC_{50} values (inverse log X where B = 50%) will then be determined and corrected to K_i values using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + (L/K_D))$, where L is the concentration and K_D is the K_D value of the radioligand. All linear and non-linear curve-fitting analyses will be performed using Prism 4.0 software.

2.3 Results The binding assay is now being conducted under the supervision of Dr. Dana E. Selley of the Department of Pharmacology and Toxicology at Virginia Commonwealth University. Both the PI's lab and Dr.

Selley's lab have fully functional binding assay facility. The assays is being pursued mainly in the PI's lab while Dr. Selley's lab will provide necessary technical support. The results for some of our ligands are summarized in Table 3 for the compounds showed IC_{50} lower than 1 uM in Ca^{2+} mobilization screening.

Table 3. [^{125}I]RANTES radio-ligand binding assay results.

Compounds	1	2	3
Ki (uM)	1.30 ± 0.12	1.68 ± 0.44	1.96 ± 0.33

As we can see from Table 3, the direct binding affinity assay for these three compounds correlated with their Ca^{2+} mobilization screening results. This further approved that using Ca^{2+} mobilization assay as pre-screening is a right decision. Therefore, in the future we are going to pursue the binding screening assays following the same route to save funds and be environmental friendly.

Task 3. Characterization of CCL5 and CCR5 expression in SV40T-immortalized human prostate epithelial cell lines of the same cellular lineage with varying tumorigenicity and metastatic capacity in vivo

This task had been finished in the first year and the results will be published in the near future (the manuscript has been published on-line).

Task 4. Investigation of the impact of chemokine receptor CCR5 antagonist on prostate cancer cell growth and progression using M12, PC-3, DU-145 and LNCaP cell lines

4.1 Anti Proliferation Assays The capacity of each synthesized compounds to inhibit the proliferation of several prostate cancer cell lines will be tested primarily as the first biological assay to evaluate their anti cancer activity.

4.2 Protocol

All cell lines, P69, PC-3, DU-145 and M12, were incubated at 37 ºC in the presence of 5% CO2. RPMI 1640 serum free media (GIBCO Invitrogen) containing 1 % L-glutamine, 0.1 % ITS (insulin, 5μg/ml; transferrin, 5μg/ml; and selenium, 5 μg/ml; Collaborative Research, Bedford) and 0.1 % gentamicin was used to cultivate all cells.

The media for DU-145 and PC-3 cell lines also included 10 % fetal bovine serum (FBS). The serum containing media for the M12 and P69 cell lines contained 5 % FBS. While the DU-145, PC-3 and P69 cells were all originally plated in media containing serum, the M12 cells were originally plated in serum containing media and then the next day were plated in serum free media and 0.01 percent epidermal growth factor (EGF). B. Anti-Proliferation Drug Assays

The effect of compounds designed as CCR5 antagonists on various prostate cancer tumor cell lines was assessed utilizing WST-1 Cell Proliferation Reagent (Roche). Prostate cancer tumor cells (DU-145, PC-3, M12 and P69) were plated out into 96 well plates (BD Falcon, VWR) at a concentration of 2000 cells per well. For the purpose of this study all cells were plated in RPMI media (GIBCO Invitrogen) containing 10 % fetal bovine serum, 1 % L-glutamine, 0.1 % ITS and 0.1 % gentamicin. The cells were placed in a total volume of 100 microliters per well and incubated overnight at 37 ºC and 5% CO2. Various concentrations of drug were plated the following day, maintaining a final total well volume of 150 microliters per well. In wells containing only cells and media or just media, a volume of 50 milliliters of PBS was added to each well. All drugs were dissolved in a minimum amount of dimethysulfoxide (Sigma-Aldrich) and the further dissolution was conducted with PBS buffer (GIBCO Invitrogen). Once the drugs were plated they were allowed to incubate with the cells for 24 hours at 37 ºC and 5% CO2.The next day 10 microliters of WST-1 Cell Proliferation Reagent (Roche) was added to each well, and incubated at 37 ºC and 5% CO2 for 3 hours. The absorbance of each well was then measured using an EL 312e Microplate Bio-kinetics Reader (BIO-TEK Instruments). Calculations of % inhibition and IC50 of each drug was performed utilizing a spreadsheet (Microsoft Office Excel 2007). The percent inhibition was calculated by subtracting the average absorbance of the cells in the

presence of drug from the absorbance of the cells with just media and then this value was divided by the difference between the absorbance of the cells without the drug and the absorbance of the media, all of which were multiplied by one hundred. The equation is, percent inhibition = [(Acells – Adrug) / (Acells – Amedia)] x 100, where A is the absorbance, cells is wells growing cells with no drug, drug is cells growing in the presence of drug and media is just media in the absence of both cells and drug.

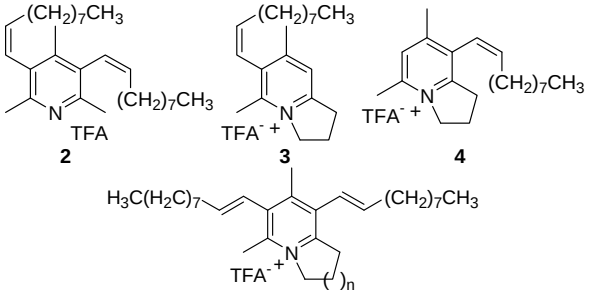
4.3 Results and Discussion

All the anibamine derivatives so far synthesized have been tested or are being tested in anti proliferation assays against PC-3, DU145, and M12 prostate cancer cell lines. Here we report the results in the way to demonstrate the Structure-Activity relationship. Therefore the numbering system is different from other Tasks.

For the deconstruction products, compound **2**, **3**, and **4** were tested. Compared with parent lead anibamine (**1**), compound **2**'s anti proliferation activity diminished drastically (IC_{50} values higher than 10 uM). This tells us that the ring system is necessary to secure the positive charge of the molecule for anti prostate cancer activity. Compound **3** and **4** showed some activity in anti proliferation assays (Table 4), but not as good as anibamine itself overall to all three cell lines. It seems that both side chains are needed to retain anti cancer activity.

So essentially the reconstruction product came back to anibamine (**1**) itself.

To further apply the method, a series of elaboration products were tested. First, compound **5** was designed to test the influence of the double bond configuration of the side chains to the activity. As shown in Table 4, double bond configuration does influence the activity somehow significantly and trans- configuration seems to be beneficial to the anti cancer activity to all three cell lines compared with anibamine. Based on this promising result, compound **6** to **7** were further designed to test the influence of the ring size to the activity. As we can see, the ring size seems to be important. While the introduction of six member ring reduced the activity dramatically, seven member ring system seems favorable to the activity against all three cell lines, especially to M12.



To summarize, the "Deconstruction-Reconstruction-Elaboration" method helped us to define the Structure-Activity-Relationship of anibamine primarily (Figure 3). It seems that overall compound **7** is showing more significant anti proliferation activity than anibamine. So we decide to apply it as our new lead compound in our future molecular design.

Table 4. Anti proliferation assay results for anibamine and compound **2-7**

Compound	IC50 (uM)		
	PC-3	DU-145	M12
Anibamine (1)	**0.37±0.04**	**0.15±0.07**	**0.50±0.08**
2	>10	>10	>10
3	0.85±0.23	4.42±1.11	2.33±0.82
4	0.14±0.05	1.21±0.31	5.9±0.60
5	**0.19±0.07**	**0.08±0.03**	**0.34±0.16**
6	0.94±0.31	4.9±1.4	6.8±1.2
7	**0.12±0.05**	**0.20±0.03**	**0.05±0.01**

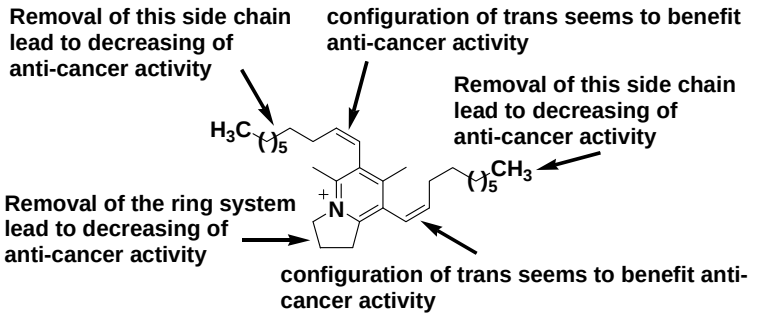


Figure 3. Initial structure activity relationship of anibamine

Task 5. Molecular modeling study

5.1. Homology modeling study of CCR5 receptor 3D model

The part of research results have been published in the Journal of Chemical Informatics and Modeling, 2009, 49(1), 120-32.

The purpose of this work is to provide a molecular modeling study template for our on-going docking study.

5.2 Small molecule construction For all the small molecules (designed and synthesized ligands), their conformation has been optimized after being constructed in Sybyl 7.0. Further dynamics simulation of each has been conducted in order to study their conformation change and possible lowest energy conformation under experimental condition. The result from this study will provide the starting point conformation of each ligand to pursue the docking study and 3D QSAR study.

5.3 3D QSAR (Quantitative Structure-Activity Relationships) Study

As proposed in our proposal, we will utilize 3D QSAR (Quantitative Structure-Activity Relationships) algorism to optimize the activities of our compounds. Some of the choices are CoMFA (Comparative Molecular Field Analysis) method and related tools such as CoMSIA. The premise of 3D QSAR is that, even in the absence of a known and structurally characterized receptor, a mathematical model of biological activity as a function of structure can be derived if a chemically and biologically meaningful 3D alignment of compounds can be made. The advantage here is that these methods ***do not*** rely upon docking the ligands into the homology model of CCR5, and therefore should provide an independent method of predicting suitability and activities for designed compounds. The structural diversity of known CCR5 antagonists are beneficial in deriving a pharmacophore model.

We now are using both binding affinity data and anti-HIV data from literature to train the QSAR models. These known CCR5 antagonists all carry high affinity to the receptor. Based on our published results and literature report, they all seem to share some common binding sites in the receptor antagonist binding domain. Therefore it is possible to define some common pharmacophore(s) among these known ligands. During the training session of our QSAR model, we have successfully defined such pharmacophores. Further comparision of these pharmacophores with the chemical structural characters of anibamine and its derivatives is being conducted right now. Following this track, we wish to publish the results in near future. Also we will adopt such result to direct our 3D QSAR study in the following year.

Key Research Accomplishments

- Another series of novel ligands have been designed based on the chemical structure of anibamine, the first nature product chemokine receptor CCR5 antagonist.

- Chemical synthesis of twenty (20) novel ligands among this series has been finished and all the ligands have been characterized fully to verify their chemical structures.

- A RANTES binding inhibition assay has been set up and the protocol has been verified using RANTES and TAK-779. The method is ready for the screening of synthesized compounds. At the meantime, a pre-screening method, Ca^{2+} mobilization assay, has been setup to test ligands primarily.

- Antiproliferation assay to evaluate the anti prostate cancer activity of the new ligands has been conducted for nine new ligands. Three different prostate cancer cell lines are adopted and the cells are treated for 24, 48, and 72 hours with varies concentrations of ligands tested. The Structure-Activity-Relationship analysis has been conducted based on the testing results.

- 3D QSAR method has been chosen to conduct our molecular modeling study. A series of known CCR5 antagonists are being training in the algorism to set up the QSAR model. Important pharmacophores have been defined and further comparision of these pharmacophores with the chemical structural characters of anibamine and its derivatives is being conducted right now.

Reportable Outcomes

One manuscript has been published so far (see Appendices) and two more manuscripts are under preparation for publication.

Two poster presentations related to this project have been presented at National and International level symposiums. They are:

1. Kendra Haney, Guo Li, John Bajacan, Amanda Richardson, Joy Ware, **Yan Zhang**. Structure-Anticancer Actvity Relationship of Anibamine, a Natural Product CCR5 Antagonist. The 239th American Chemical Society National Meeting, March 21-25 San Francisco, California, USA.

2. Kendra Haney, Guo Li, John Bajacan, Amanda Richardson, Joy Ware, **Yan Zhang**. Structure-Anticancer Actvity Relationship of Anibamine, a Natural Product CCR5 Antagonist. VCU School of Pharmacy Research and Career Day, Octobor 2009, Richmond, VA.

One provisional invention disclosure related have been filed:

Title: Anibamine and its analogues as novel anti-HIV and anti-cancer agents
Inventor: Yan Zhang
VCU Invention No. ZHA-10-024

One Master Degree student (Kendra Haney) has graduated after finishing a research project related.

One Master Degree student (Shilpa Singh) is expected to graduate in the Summer of 2010 upon the completion of thesis based on this project.

One Postdoctoral Associate (Guo Li) has been trained under the support of this award and has moved onto another postdoctoral training site in Purdue University.

One Postdoctoral Associate (Shashidhar Akubathini) is being trained under the support of this award and is ready to apply for industrial chemist position.

Conclusion

The major focus of the research project is the syntheses of the ligands we designed as chemokine receptor CCR5 antagonists with novel structural feature(s) derived from anibamine, the first natural product as a CCR5 antagonist. The biological characterization of these ligands for anti prostate cancer activity is being conducted. Once biological test results are available, Structure-Activity-Relationship (SAR) analyses will be conducted to identify the structural features for the next generation lead compound(s). When enough data is available, 3D QSAR studies will be performed.

In the second year we have finished the chemical synthesis of another twenty novel ligands that are designed based on "Deconstruction-Reconstruction-Elaboration" method. A RANTES-binding inhibition assay protocol has been set up and ready to test our drug candidates. A Ca^{2+} mobilization assay as the pre-screening method has been set up in the second year. Other biological screening includes the test of capacity of these novel compounds to inhibit proliferation and/or apoptosis by the human prostate cancer cell lines M12, PC-3, DU-145 and LNCaP has been conducted to evaluate their efficacy. For the molecular modeling study we have setup the training set of compounds from literature and have successfully defined important pharmacophores for these ligands. Further comparision of these pharmacophores with the chemical structural characters of anibamine and its derivatives is being conducted right now.

In the last year of this project (no cost extension period), we will focus on the structure modification/elaboration part of the proposal by introducing more varieties of substitutions at the two side chains by following the "Lipinski's rule of 5" for drug-like compounds. Further biological screening will start from the Ca^{2+} mobilization assay to the radioligand binding assay, to in vitro anti prostate cancer tests. We wish to identify a promising lead compound with novel chemical and structural features as well as high therapeutic indices to serve our long-term goal in finding a potent anti prostate cancer agent.

The medical product expected from this project in long term is discovery of a novel and potent anti prostate cancer agent with high therapeutic index.

Appendices

Publication Reprint

Xueping Zhang, Kendra M. Haney, Am anda C. Ri chardson, Eden W ilson, David A. Gewirtz, Joy L. Ware, Zendra E. Zehner, **Yan Zhang**. Anibamine, a Natural Product CCR5 Antagonist, as a Novel Lead for the Developm ent of Anti Prostate Cancer Agents. *Bioorg. Med. Chem. Lett. 2010, in press.*

Invention Disclosure

Title: Anibamine and its analogues as novel anti-HIV and anti-cancer agents

Inventor: Yan Zhang

VCU Invention No. ZHA-10-024

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Anibamine, a natural product CCR5 antagonist, as a novel lead for the development of anti-prostate cancer agents

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ABSTRACT

Accumulating evidence indicates that the chemokine receptor CCR5 and the chemokine CCL5 may be involved in the proliferation and metastasis of prostate cancer. Consequently, chemokine receptor CCR5 antagonists could potentially act as anti-prostate cancer agents. As the first natural product CCR5 antagonist, anibamine provides a novel chemical structural skeleton compared with other known antagonists identified through high-throughput screening. Our studies demonstrate that anibamine produces significant inhibition of prostate cancer cell proliferation at micromolar to submicromolar concentrations as well as suppressing adhesion and invasion of the highly metastatic M12 prostate cancer cell line. Preliminary in vivo studies indicate that anibamine also inhibits prostate tumor growth in mice. These findings indicate that anibamine may prove to be a novel lead compound for the development of prostate cancer therapeutic agents.

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Prostate cancer is the most common non-cutaneous solid cancer occurring amongst men in the USA, and the second most common malignant cause of male death worldwide.[1] Current therapies remain limited to surgery, radiation, and/or androgen ablation.[2] Recent investigations indicate that there is a relationship between some inflammatory processes and cancer, specifically, prostate cancer development.[3–13] For example, the prostate cancer cell lines PC-3, DU145, and LNCaP express the chemokine CCL5 (RANTES) and the chemokine receptor CCR5. Furthermore, the chemokine receptor CCR5 antagonist, TAK-779 inhibited CCL5-induced proliferation of these prostate cancer cell lines.[12] Levels of CCL5 and CCR5 are also reported to be greater in prostate cancer specimens than in benign hyperplasia.[13] Collectively these findings in both patient-derived specimens and prostate cancer cell lines suggest that development of the appropriate chemokine receptor CCR5 antagonists could provide a novel prostate cancer therapy.

Anibamine (Fig. 1), a novel pyridine quaternary alkaloid recently isolated from *Aniba* sp., was found to bind to CCR5 with an IC_{50} of 1 μM in competition with ^{125}I-gp120, an HIV viral envelop protein.[14] Thus far, anibamine is the first known natural product acting as a CCR5 antagonist. While the chemokine receptor CCR5 has mainly been targeted in HIV therapies since it was first cloned more than a decade ago,[15–21] CCR5 antagonists could provide a novel therapeutic approach for prostate cancer treatment through the inhibition of CCL5-induced cell proliferation.

Anibamine has a novel structural skeleton compared to other CCR5 antagonists identified through high-throughput screening. Considering the binding affinity to CCR5 of other original lead

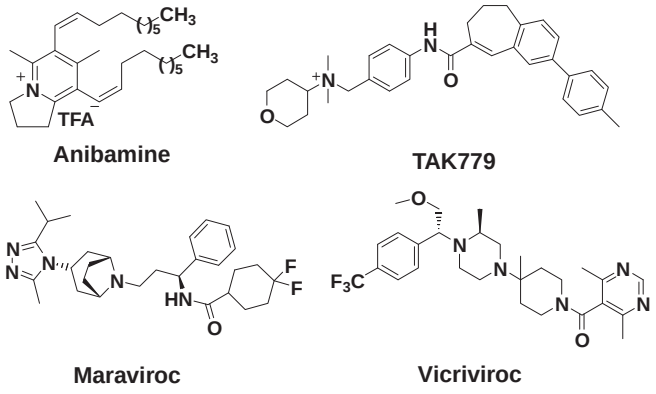

Figure 1. Anibamine and some known CCR5 antagonists.

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compounds,[22–24] the inhibitory binding affinity of anibamine at 1 μM to CCR5 appears quite promising.

Recently, the total synthesis of anibamine has been reported by one of our laboratories.[25] The development of this synthetic pathway provides the opportunity for generating anibamine derivatives in order to explore their structure–activity relationships as CCR5 antagonists. The binding of anibamine to the chemokine receptor CCR5 has been characterized and compared with that of other CCR5 antagonists in different homology models of CCR5.[26] The binding pocket of anibamine shares some common features with other high affinity CCR5 antagonists, suggesting binding to similar binding sites. The current studies were designed to explore the utility of developing anibamine as a novel lead compound against prostate cancer.

As indicated previously, the expression of CCL5 and CCR5 has been observed in various prostate cancer cell lines, including PC-3, DU145, and LNCaP.[12,13] Expression of CCR5 and CCL5 mRNA was quantitated via qRT-PCR in the highly metastatic M12 prostate epithelial cell line, as well as in its non-tumorigenic parental cell line P69.[27] The results, shown in Figure 2, indicate that while both genetically related sublines express CCR5, CCL5 expression was evident in the M12 tumorigenic subline but was barely detectable in the parental p69 line. From our results, the relatively elevated levels of CCL5 in the metastatic M12 cell line compared to the non-tumorigenic parental p69 line suggest that CCL5 and its receptor CCR5 could be involved in prostate cancer metastatic progression, providing additional support for the potential value of targeting the chemokine receptor CCR5 in prostate cancer.

Previously, M12 cells were shown to have a very high invasive ability.[27] It is also known that adhesion and invasion are important steps that further promote prostate tumorigenesis and metastasis. The growth inhibitory properties of anibamine were evaluated in the prostate cancer cell lines, PC-3, DU145, and M12. Results of these assays are summarized in Figure 3. Anibamine was observed to interfere with prostate cancer cell growth in a dose-dependent manner at micromolar to submicromolar concentrations in all three cell lines. The observations that anibamine can inhibit the invasion and adhesion of M12 cells support the possibility that anibamine may have anti-metastatic properties against prostate cancer.

In invasion and adhesion assays, the addition of anibamine inhibited M12 invasive ability by 42–65% (Fig. 4) depending on the dose and M12 adhesion up to 26% (Fig. 5). No additional effects on adhesion were evident at higher concentrations (data not shown).

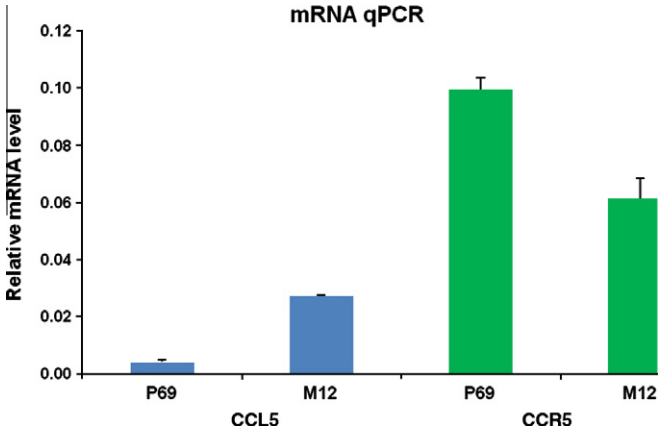

Figure 2. Differential expression of CCL5 and CCR5 in isogenic P69 and M12 prostate cancer sublines. SYBR-based qRT-PCR was performed with total RNA extracted from P69 and M12 sublines as described in Materials and methods. The *Y*-axis represents the relative mRNA level of CCL5 or CCR5 normalized to RNU48 as an internal control. The standard error of the mean is shown as error bars. Student's *t*-test indicates a significant difference with a *P*-value <0.001 for both CCL5 and CCR5.

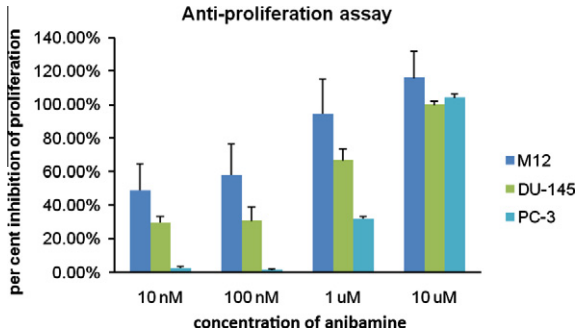

Figure 3. Inhibition of prostate cancer cell proliferation by anibamine. Three prostate cancer cell lines, M12, PC-3, and DU145 were exposed to a series of concentrations of anibamine. Proliferation was assessed using the WST-1 cell proliferation reagent (Roche).

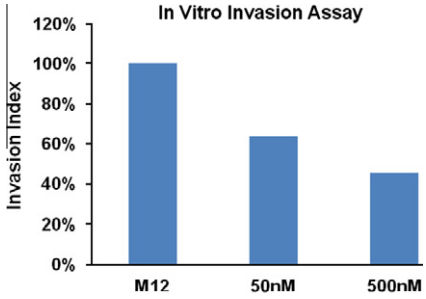

Figure 4. Effect of anibamine on invasive capability of M12 cells. Equal numbers of M12 cells were plated in Transwell chambers ± anibamine at the indicated concentrations as described in Materials and methods. Filters were coated with 1:10 diluted lrECM prior to cell plating. Medium containing 20% FBS, EGF (20 ng/ml) and 5 ng/ml CCL5 was added as a chemo-attractant to the lower chamber. Bars indicate standard error. ANOVA testing indicates a significant difference with a *P*-value <0.001.

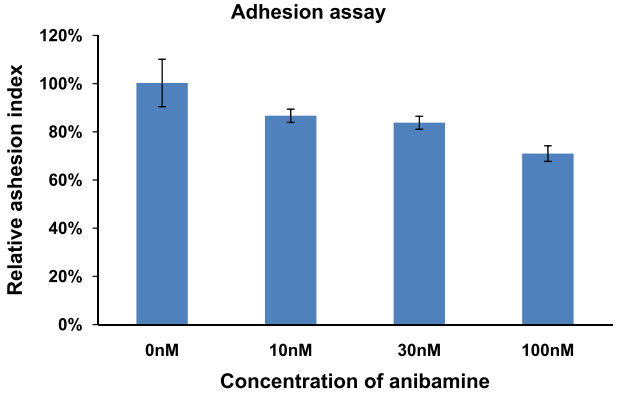

Figure 5. Effect of anibamine on M12 cell adhesion. Equal numbers of M12 cells were pre-treated ± anibamine at the indicated concentrations for 24 hours and were then plated in 96-well cells coated with diluted lrECM as described in Materials and methods. The *X*-axis represents the concentration of anibamine, while the *Y*-axis represents the relative adhesion index normalized to the M12 control without drug. Bars indicate standard error. ANOVA test indicates a significant difference with a *P*-value of <0.001 ($F = 26.8$).

Further, M12 cells embedded in lrECM gels were studied to assess the effect of anibamine on tumor cell morphology. As shown in Figure 6, the M12 subline displayed a disorganized mass of cells when grown in 3D (which is in agreement with its metastatic character); the addition of anibamine reverted M12 cells to spheroid-like

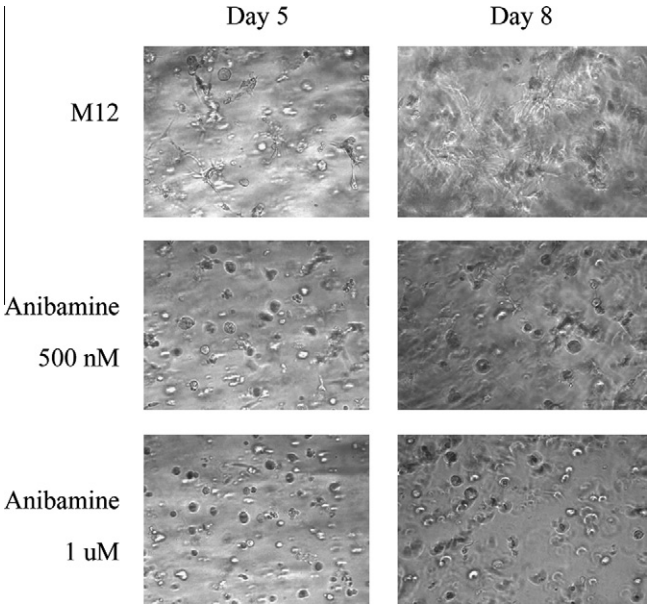

Figure 6. Growth properties of M12 cells ± anibamine in 3D lrECM gels. M12 cells (1×10^5) were mixed with 100 μl of undiluted lrECM gel ± 500 nM or 1 μM anibamine and then plated in 96-well plate as described in Materials and methods. Light microscopy images were taken from cultures at day 5 and 8 as indicated. Magnification is at 10×.

structures referred to as acini. These observations further support the premise that anibamine indeed could potentially inhibit prostate tumor metastasis.

After that, immunohistochemical staining for relevant cell proteins coupled with confocal microscopy was conducted to better examine the morphological differences displayed by these cells when grown embedded in lrECM gels. The M12 subline, which shows high expression of vimentin, spread throughout the lrECM as a disorganized mass, which reflects the highly tumorigenic/metastatic behavior of these cells when injected into male, athymic nude mice.[28] Interestingly, vimentin gene expression declined with the addition of anibamine (Fig. 7). In addition, while the expression of α6- and β1-integrins was quite disorganized in M12 cells. The addition of anibamine reverted the disorganized mass of cells to an acinus with a distinct luman displaying basal polarization of α6- and β1-integrin as shown previously for the parental, benign P69 cells.

As with the development of any new class of drugs, it was critical to determine whether these compounds could be used at concentrations that are not toxic to normal cells. For our initial screening studies, we examined hemolysis of sheep red blood cells by anibamine, since this was thought to be a possible limitation on the use of this class of agents.[29] Our result indicates that no toxicity was observed in this assay below or at a concentration of 1 μM (Fig. 8), which would support the potential selectivity of this agent.

In addition, preliminary data from an on-going in vivo analysis suggests that anibamine can reduce the subcutaneous growth of M12 tumor cells in athymic nude mice. In the three mice with subcutaneous M12 tumors, four days after four injections of anibamine, the size of the tumors was 321.4 mm^3, 80.0 mm^3, and 202.2 mm^3, respectively, averaging 201.2 ± 69.7 mm^3. In contrast, the size of the tumors injected with the solvent control was 421.6 mm^3, 182.6 mm^3, and 384.6 mm^3, respectively, averaging 329.6 ± 74.3 mm^3. Thus anibamine did appear to reduce the rate of growth of the M12 tumors by roughly 50% (Fig. 9). Such observation that anibamine can reduce the subcutaneous growth of M12 tumor cells in athymic nude mice support the premise that

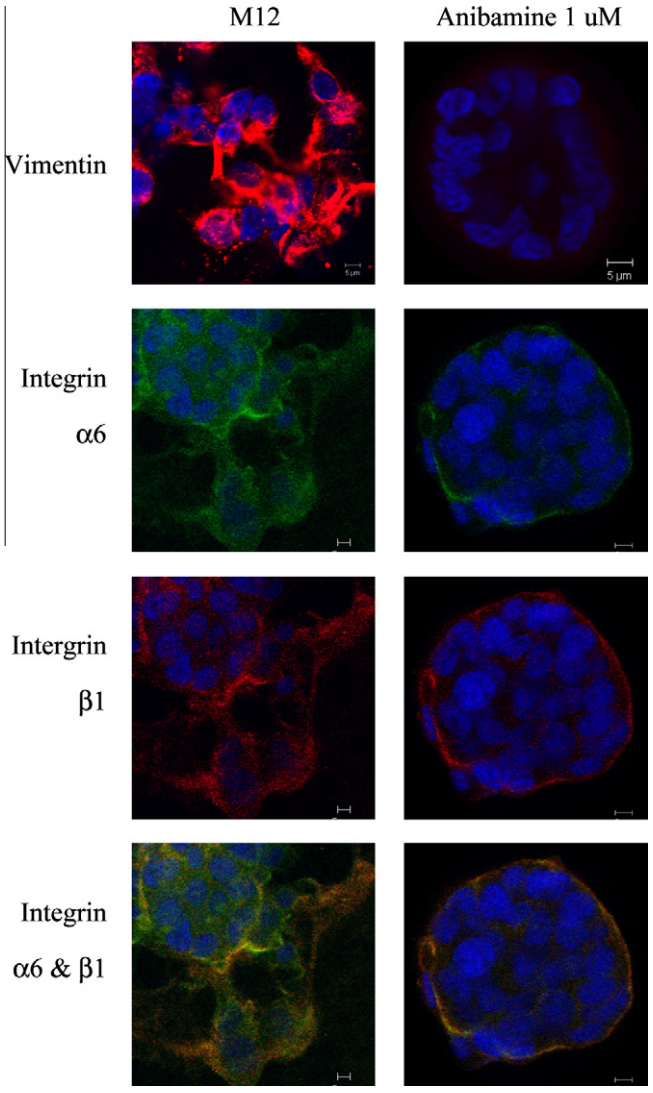

Figure 7. Comparison of content and localization of vimentin and integrin within the morphological structures formed by the M12 prostate sublines ± anibamine grown embedded in lrECM gels. Confocal immunofluorescence microscopy of structures formed at day 8 stained with anti-bodies to vimentin (red, top panel), α6-integrin (green) and β1-integrin (red) as indicated. The overlay of α6β1-integrin is shown on the bottom panel. All pictures are taken at a magnification of 63× and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue) as discussed in Materials and methods.

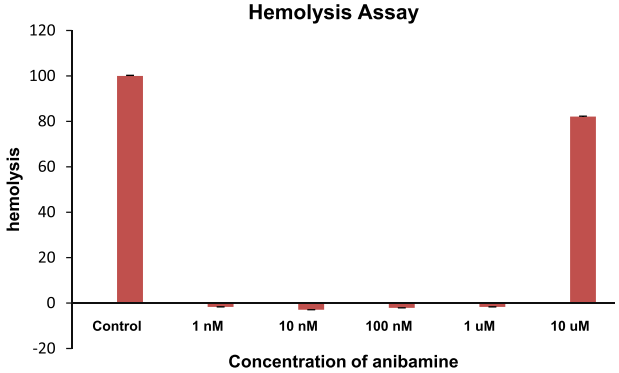

Figure 8. Assessment of red blood cell hemolysis by anibamine. Sheep red blood cells were exposed to anibamine in PBS for 60 min. Samples were centrifuged and the absorbance of the supernatant determined at 540 nm. Lysis in distilled water was used as a positive control.

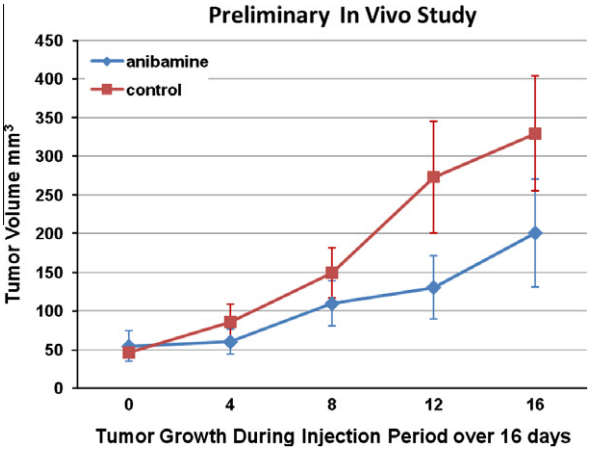

Figure 9. Influence of anibamine on prostate tumor growth in vivo. Six athymic nude mice were injected subcutaneously with 2000 M12 cells. After the tumors became visible, the mice were injected intravenously via a lateral tail vein with 0.3 mg/kg of anibamine or with 0.3 mg/kg of saline over a 16 day period for four injections (once every fourth day). The tumor size was recorded accordingly as the number of day right after the first injection.

anibamine and/or its derivatives could prove to have utility in the treatment of prostate cancer.

In summary, anibamine showed significant inhibition of prostate cancer cell proliferation at 1 μM and lower concentrations while direct hemolysis was not evident until an approximately 10-fold higher concentration. Anibamine also suppressed the invasive and metastatic properties of M12 cells and compromised the growth of these tumors in vivo. Overall these preclinical studies suggest that anibamine could have a reasonable therapeutic index, supporting the potential utility of this compound as the lead for future drug design and development.

One reservation is that the calculated log K_{ow} for anibamine is 9.1,[29] which indicates that its lipophilicity is significantly higher than the value set forth by 'Lipinski's rule of 5' for drug-like compounds.[30] In comparing the chemical structure of anibamine with other known CCR5 antagonists (Fig. 1), a major difference is that the anibamine side chains are simple, undecorated, aliphatic chains. Therefore, further drug development for CCR5 antagonists based on the chemical structure of anibamine, the first natural product with high binding affinity to the CCR5 chemokine receptor, may lead to a new type of therapeutic agent for metastatic prostate cancer therapy. Further studies of anibamine and its analogs should also serve to clarify the mechanisms by which targeting the chemokine receptor CCR5 may suppress metastatic processes of prostate cancer cells.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.003.

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Docket No. ZHA-10-024 **ASSIGNMENT**

WHEREAS, Yan Zhang, Ph.D. **(INVENTOR)** being a member of Virginia Commonwealth University, has invented certain new and useful improvements in an invention entitled: *"Anibamine and its analogues as novel anti-HIV and anti-cancer agents"*, VCU 10-024 **(INVENTION).**

WHEREAS, Virginia Commonwealth University **(UNIVERSITY),** a corporate instrumentality of the Commonwealth of Virginia, is desirous of acquiring certain rights thereunder; and **INVENTOR** will transfer said rights directly to the **UNIVERSITY;**

WHEREAS, the **INVENTION** was made using facilities and resources controlled by the **UNIVERSITY;** and

WHEREAS, Virginia Commonwealth University's Intellectual Properties Policy, which applies to me as an member of Virginia Commonwealth University pursuant to Section 23-4.3.B of the Code of Virginia provides, among other things, that inventions made using facilities and resources controlled by the **UNIVERSITY** become the property of the **UNIVERSITY,** and that **UNIVERSITY** member-inventors are obligated to assign their rights in the **INVENTION** and any patent applications) and any patent(s) issued thereon to the **UNIVERSITY;**

WHEREAS, the Virginia Commonwealth University Intellectual Property Foundation **(FOUNDATION)** is the lawful, not-for-profit entity by which the **UNIVERSITY** commercializes intellectual property;

NOW, THEREFORE, for the sum one dollar ($1.00) and other good and valuable consideration, receipt of which is hereby acknowledged, I agree to and do hereby sell, assign and transfer unto said **UNIVERSITY** the entire right, title, and interest in and throughout the United States of America (including its possessions and dependencies) and all countries foreign thereto, in and to said **INVENTION** (whether patentable or not), and any and all patents (including reissues and extensions thereof), of any country, which have been or may be granted on said **INVENTION** or any part thereof, or any divisional, substitute, continuation in whole or in part, renewal, reissue or other patent application based thereon, and based upon said **INVENTION,** together with the right of said **UNIVERSITY** to apply for such patent in its own name in all countries of the world where such is permissible by law, and the right to claim the benefit of the priority right provided by the International Convention of 1883, as amended to date, and any such priority right;

TO BE HELD AND ENJOYED BY said **UNIVERSITY,** its successors and assignees, to the full ends of the respective terms for which said patents or any of them have been or may be granted as fully and entirely as the same would have been held and enjoyed by me had no sale and assignment of said interest be made;

AND I do hereby authorize and request the Commissioner of Patents of the United States of America to issue any and all United States Patents which may be granted upon said **INVENTION** or any part thereof, to said **UNIVERSITY;**

AND I hereby agree for myself, and for my heirs, executors and administrators, to execute without further consideration any further lawful documents and any further assurances, and any divisional, continuation in whole or in-part, substitute, renewal, reissue, or other applications for patents for any country that might be deemed necessary by said assignee fully to secure to said assignee its interest as aforesaid in and to said **INVENTION** or any part thereof, and in and to said several patents or any of them;

AND in addition I agree that any and all royalties, rents, payments or any receipts from the sale, assignment, transfer licensing or use of said **INVENTION,** whether patented or not, which are received by the said **FOUNDATION** shall be the property of the **FOUNDATION** with the understanding that I will receive a percentage of said royalties, rents, payments, or receipts pursuant to and in accordance with the Virginia Commonwealth University Intellectual Properties Policy as adopted August 12, 1999 and amended May 15, 2009.

AND I do hereby covenant for myself and my legal representatives and agree with said **UNIVERSITY,** its successors and assignees that I have granted no right or license to make, use or sell said **INVENTION** to anyone except said **UNIVERSITY,** that prior to the execution of this deed our right, title and interest in said **INVENTION** had not been otherwise encumbered, and that I have not executed and will not execute any instrument in conflict herewith.

Executed this 6 day of April 2010.

Creator / Inventor

Yan Zhang, Ph.D.

VIRGINIA COMMONWEALTH UNIVERSITY

Ivelina S. Metcheva, Ph.D., MBA
Director, VCU Tech Transfer

INTELLECTUAL PROPERTY FOUNDATION

Ivelina S. Metcheva, Ph.D., MBA
President, VCU-Intellectual Property Foundation

Commonwealth of Virginia
City of Richmond, to-wit:

Subscribed and sworn to before me this 6 day of April, 2010.
Witness my hand and official seal.

T. Allen Morris

Notary Public

T. ALLEN MORRIS
NOTARY PUBLIC
Commonwealth of Virginia
Reg. #7104856
My Commission Expires: 5/11/2011